THE EMBO JOURNAL

Manuscript EMBO-2015-93084

Mfn2 deficiency links age-related sarcopenia and impaired autophagy to activation of an adaptive mitophagy pathway

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Review timeline:

Submission date:	30 September 2015
Editorial Decision:	30 October 2015
Revision received:	27 April 2016
Editorial Decision:	11 May 2016
Revision received:	25 May 2016
Accepted:	27 May 2016

Editor: Andrea Leibfried

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 30 October 2015

Thank you for submitting your manuscript to us. I have now received reports from all referees, which you will find enclosed below.

As you will see, the referees appreciate your study. However, they do not think that the advance and insight currently provided is sufficient for publication in The EMBO Journal. They note that the novelty is limited and that your claims need much further support. Importantly, it is unclear whether the observed effects are rather due to Mfn2 loss of function in WAT, liver or heart, as the analyzed knock-out is unspecific.
Given the constructive comments provided, I can invite you to provide a revised version of your manuscript, addressing all concerns of the referees.
Clearly, analyzing a more specific knock-out model is not feasible within one round of revision. But you need to address the specificity concern (see especially the reports from ref #1 and #3) convincingly to support that the phenotype of your KO mice is the consequence of a mitochondrial dysfunction only in skeletal muscles and not in other tissues. Furthermore, more insight into how aging leads to a decline in Mfn2 is needed, and how general the decline in the different mouse tissues is. Also, the mitochondrial phenotype, the metabolic changes, and the muscle function changes need a better characterization, as do the autophagy alterations.

Please note that I will require strong support from the referees on a revised version for further consideration here. The revision thus demands a lot of work, with uncertain outcome. So please

consider your options carefully. I can extend the revision time to 6 months, should that be helpful. Please note that in the case that a muscle-specific effect is not supported upon further analyses, you should consider going elsewhere. Please contact me if you have any questions regarding the revision of your manuscript.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

The manuscript of Sebastian and co-authors describes the role for Mfn2 in sarcopenia and age-related metabolic adaptations. The authors have generated a muscle-specific Mfn2 knockout mice to address the relevance of mitochondrial dynamics in ageing sarcopenia. The authors have well characterized the KO mice at morphological, biochemical and metabolic level. The authors propose a mechanism where the reduction/ablation of Mfn2 leads to deficient mitophagy which in turn results into the accumulation of dysfunctional mitochondria. Damaged mitochondria produce ROS that activate a retrograde signal via HIF1-BNIP3 to induce mitochondrial removal via mitophagy. The present work is a well designed study with interesting findings. This enthusiasm is lowered by the fact that the authors already have characterized Mfn2 knockout mice in young age and have found a metabolic imbalance. The metabolic changes observed may indirectly induce the precocious ageing phenotypes observed in these KO mice. However, this work better clarifies some of the contradictory results present in literature. In fact, there are several reports connecting sarcopenia with dysregulation of mitochondria quality control. However, mitochondrial dynamic protein levels have been found either downregulated (Ibebunjo et al. 2012) or unchanged (Leduc-Gaudet et al. 2015). Thus, the causal link between sarcopenia and unbalanced mitochondrial fusion/fission is not clear. Therefore, this manuscript provides interesting data supporting a causative role of mitochondrial dynamics to age-related muscle loss. However, some findings are preliminary and need further experiments to sustain author's claims. The authors should consider the following points.

Major Points:
Figure 1. The authors start the paper demonstrating that aging is characterized by a progressive reduction of Mfn2 protein levels (Figure 1). The authors should check whether this decrease is consequent to a transcriptional-dependent suppression or protein turnover. Moreover, It would be of interest to know whether this decrease is present in other muscles a part from gastrocnemius such as soleus, EDL, quadriceps or Tibialis Anterior. The densitometric quantification of Mfn2 should be normalized for mitochondrial proteins in order to detect a relative decrease of Mfn2 compared to mitochondrial content.

Point 2. One critical point of this genetic model is that the deletion of Mfn2 looks not specific. Indeed authors described that Mfn2 is reduced in tissues like WAT, liver and heart. Therefore, it is difficult to say which tissue contributes to the age-related metabolic alterations. It would be important to monitor mitochondrial complex activity and mitochondrial respiration also in WAT, Liver and heart in order to have an idea whether mitochondria are compromised in these tissues.

Point3, Figure 2. During ageing it has been reported an increase of mtDNA mutation. The authors should check whether there is an increase of COX negative/SDH positive fibers in Mfn2 KO compared to controls. The authors claimed that Mfn2 KO muscles contain giant mitochondrial. The EM pictures shown in panel 2G are small and it is difficult to appreciate mitochondrial morphology. Larger images are encouraged. However, looking also at the panels 2H, it does not appear that mitochondria are giant but more likely are swollen. Moreover, it would be important to characterize the fission machinery. Is there any changes in DRP1/fis1/Mff proteins that would account for the giant mitochondria mentioned by the authors? What about Mfn1 and OPA1 levels in aged Mfn2 KO mice? Finally, the quantification of Mitochondrial complex activity would be an important addition to the characterization of mitochondria when mfn2 is absent.

Point 4. Figure 3. it would be interesting to monitor some of the pathways involved in glucose

uptake in muscles such as Akt, AMPK, TBC1D1, AS160.

Point 5. Figure 4. During ageing there is a progressive loss of motorneurons leading to denervation. This process mainly happens in fast fibers and is partially counteracted by reinnervation of slow motorneurons. Nerve sprouting and fiber-type grouping are typical features of ageing sarcopenia. Did the authors reveal an increase of type I fibers and/or type I-grouping? Experiments represented in Figure 4 correlate Mfn2 abaltion with the reduction of muscle mass and function. Figure 4G shows that old Mfn2 KO mice have reduced muscle performance on a treadmill. However, this type of test does not discriminate between the performance of skeletal or cardiac muscles. It is worth to underline that these mice show a reduction of Mfn2 in both heart and skeletal muscles. Sarcopenia can be defined as the reduction of both muscle force and muscle mass. Importantly, the reduction of muscle force is faster than the reduction of muscle mass. Therefore, the analyses of muscle force in Mfn2 KO would be more informative than exercise performance. Muscle mass is controlled by the balance between protein degradation and protein synthesis. While the authors attempt to investigate the activation of proteolytic pathways responsible of muscle loss, protein synthesis is superficially studied. Indeed, western blot analysis of phospho- and total-4EBP1 is not sufficient to determine the level of protein synthesis. A better investigation of Akt-mTOR pathways is required. Finally, protein synthesis should be measured in sarcopenic muscles by using puromycin incorporation.

Point 6. Figure 5. The authors excluded an involvement of ubiquitin-proteasome system based on the expression levels of atrogin1 and MuRF1 and on proteasome activity, determined in vitro. However, we now know that many other ubiquitin ligases are involved in muscle atrophy. Moreover, the proteasome activity depends on the level of the poly-ubiquitinated proteins unless proteasome in inhibited. Authors should check whether the amount of poly-ubiquitinated proteins is increased in Mfn2 KO mice. A western blot for Lys48 poly-ubiquitinated proteins and its quantification would better complement the analyses of the Ubiquitin-proteasome system.
The authors have used C2C12 to monitor autophagy flux. The authors should confirm the in vitro data and monitor autophagy flux in vivo by colchicine or leupeptin treatments. The changes of p62 level displayed in panel 5B may reflect transcriptional changes. The authors must check the mRNA levels of p62/SQSTM1 in Mfn2 KO. In panel 5D the authors found an identical increase of LC3II band after bafilomycin treatment in control and Mfn2 KO cells. This does not happens for p62, NBR1 and BNIP3. The authors' claim that autophagy flux is reduced is incorrect. The right explanation of these data is that non-selective autophagy is maintained but this is not the case for the selective autophagy that requires the adaptor proteins p62, nbr1 and bnip3 for the selective removal of damaged proteins/mitochondria. Thus, Mfn2 KO mice upregulate non selective autophagy because the selective autophagy is impaired. It would be important to check LC3II, P62 and BNIP3 levels on purified mitochondria from muscles of Mfn2KO and controls in presence or absence of Colchicine. The experiments on purified mitochondria from myotubes shown in Fig 5G support the hypothesis of an impairment in selective autophagy but does not consider the flux.

Figure 7F recapitulates the mechanism proposed in this work. The figure is representative of the data but the last panel is unclear. To my opinion, in this panel the line connecting Mitochondrial quality control to mitochondria should be removed, mitochondria should become swollen, the arrow coming out from mitochondria and the world ROS should be enlarged and all the arrows connecting HIF1a- Bnip3-mitchondrial quality control removed as well as the text (HIF1a, BNiP3). This new version would better explain the message that when quality control is impaired then dysfunctional mitochondria accumulate leading to enhanced ROS production. Finally, the sentence in the middle panel "Impaired metabolic homeostasis sarcopenia and muscle dysfunction" should be removed since it is also present in the last panel and makes confusion. In fact, in healthy ageing the HIF1a loop should work in order to minimize the age-related changes. Maybe the authors can use "physiological age-related muscle loss" in healthy ageing and "sarcopenia and frailty" in Unhealthy ageing.
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Minor Points:
-Figure 1B: The comparison between genes deregulated in old Mfn2 KO mice versus old wild-type is missing.
-Figure EB2A: Western blots are not shown and should be provided.
-Figure 5B: Western blot for tubulin is oversaturated
-Figure 5G: Western blot for Parkin was cropped at the level of the band.
The reduction of autophagy in aged muscles have been shown in the work of Carnio et al. Cell

Reports 2014 in which authors showed that autophagy is decreased in aged humans and mice, that autophagy inhibition worsens muscle atrophy and force generation and that restoring autophagy in aged muscle ameliorates muscle mass and innervation. Therefore, this paper should be mentioned by the authors in introduction and discussion.

Referee #2:

EMBO J (18 oct 2015)
Mfn2 deficiency links age-related sarcopenia and impaired
autophagy to activation of an adaptive mitophagy pathway

1- General summary and opinion about the principle significance of the study, its questions and findings
In this paper, the authors show that during skeletal muscle aging Mfn2 is a key molecule to regulate autophagy and mitochondria quality control. They show that Mfn2 depletion triggers an impairment of mitophagy associated with a gene signature related to aging, thereby leading to alterations in metabolic homeostasis and sarcopenia. Interestingly, they also show that these effects are attenuated by the Mfn2-depletion-dependent activation of mitophagy through an alternative pathway driven by HIF1a activation. This paper is very coherent in the presentation of the outstanding questions, the state of the art and the results obtained. The experiments are well designed and performed, both quantitatively and qualitatively. To sum up, the work presented in this paper is very original and particularly since it adds new functions to the ones already known the mitochondrial dynamic protein Mfn2, thereby extending our knowledge on mitochondrial involvement in crucial cellular and physiological pathways.

2- specific major concerns essential to be addressed to support the conclusions

How can authors explain that Mfn2 expression level is decreased in aging, and in particular in highly oxidative muscle fibers? Did they check if Mfn2 expression is regulated by one of the common inducers of senescence (p53, pRb, ...), or affected by ROS ?

Moreover, one experiment is missing to conclude that Mfn2 is involved in the aging process: the overexpression of Mfn2 in old mice, or at least in senescent primary cells to determine if there is a rescue in the different phenotypes observed. Furthermore, the in vitro model shall be better characterized to understand the molecular mechanisms involved. For instance, most of the in vitro experiment are performed in C2C12 cells, but the use of primary fibroblasts could be more relevant to determine if Mfn2 depletion also triggers or accelerates cellular senescence, by studying the usual markers of senescence like telomere shortening, p53, pRb, p16 or p21 activation, SA b-gal activity,...

Along the same line, the aging phenotype is caused by which of the consequences of Mfn2-ablation (or reduction)? Is it ROS production, autophagy reduction, decreased ER-mitochondria tethering (and hence autophagy) or something else? For instance, it has been shown that the inhibition of autophagosome formation by treatment with 3-methyladenine decreases the number of SA-β-GAL-positive cells upon treatment to induce senescence (Patschan et al. 2008). Is autophagy or ROS inhibition with 3MA or NAC additive to Mfn2 depletion?

In figure5D there are some issues. First, in the Mfn2 KD LC3 or p62 seem to be unaltered, whereas in vivo the differences between wt and Mfn2-/- were clear... How do the authors explain this discrepancy? Moreover, the quantification of BNIP3 and NRB1 levels in the experiment w/o bafilomycin does not seem to be a faithful representation of the shown WB.

In figure 6, NAC is used to conclude that the ROS-dependent HIF1 pathway is important to maintain a certain level of mitophagy and counterbalance the Mfn2-depletion-dependant mitophagy decrease. However, by closely looking at the first WB, it seems that there is a huge increase in mitochondrial Parkin levels upon NAC treatment, which is not mirrored in the presented quantifications. Thus, to conclude that NAC affects the autophagic flux, Bafilomycin or chloroquine must be added. Finally, other experiments are needed to conclude that ROS production upon Mfn2 ablation is protective. What about sarcopenia, or mice activity?

Referee #3:

Summary:

The current study by Sebastián et al. uses a model of Mitofusin 2 (Mfn2) deficiency to examine how this protein contributes to the accumulation of damaged mitochondria in skeletal muscle associated with aging and metabolic disease. The authors suggest that Mfn2 protein expression is reduced in skeletal muscle of old mice (22 months). Microarray analysis showed that many genes were similarly regulated in muscle of old control and young Mfn2 deficient as compared to young control animals (6 months). Although Mfn2 deficiency did not alter lifespan, the animals displayed many hallmarks of mitochondrial dysfunction including reduced mitochondrial density, increased ROS production and observation of large swollen mitochondria. The mitochondrial dysfunction observed in Mfn2 deficient animals was associated with development of more severe metabolic disease in old animals including reduced glucose tolerance and insulin sensitivity. Mfn2 deficient animals had smaller muscle fibers, which resulted in a reduction in muscle weight in young but not old animals. Furthermore, Mfn2 deletion was associated with reduced running capacity in old but not young animals. The authors suggest that Mfn2 knockout mice had reduced muscle autophagy that leads to atrophy and mitochondrial dysfunction. As a result, HIF1α protein levels are increased to up-regulate Bnip3-mediated autophagy.

This work confirms that aging is associated with the accumulation of damaged mitochondria and a reduced ability to repair and degrade damaged mitochondria in muscle which results in metabolic dysfunction, loss of muscle mass and activation of mitochondrial quality control pathways. Based on the data presented, it is unclear what the specific role of Mfn2 is in the regulation of this process. Furthermore, the authors are unable to uncouple the muscle-specific and systemic effects of Mfn2 deficiency on development of metabolic disease, muscle mass and muscle function. This work does not provide novel insights into the role of Mfn2 in mitochondrial homeostasis or fusion in skeletal muscle or how mitochondrial quality control is reduced by aging.

Major Concerns:

1. The authors describe the animals as "muscle Mfn2-deficient mice", however they have previously demonstrated that Cre-mediated deletion of Mfn2 driven by the MEF2C promoter also occurs in many tissues including Liver, adipose tissue and brain. This group has previously demonstrated that liver specific (Albumin-Cre) deletion of Mfn2 is sufficient to cause metabolic dysfunction. A well-characterized muscle-specific promoter driven Cre such as human skeletal muscle actin 1 (hACTA1) or muscle creatine kinase (MCK) should be used. Young animals examined in this study do not display a strong metabolic phenotype indicating that systemic insulin resistance may contribute to this phenotype. Notably, the insulin resistance phenotype in older Mfn2KO mice is likely secondary to increased weight gain. The authors should also indicate what were used as control mice.
2. In Fig. 1B, the authors claim that Mfn2KO muscle show an aging signature expression profile. However, only a very small number of genes actually overlap with aged muscle. If Mfn2 indeed is involved in muscle aging, young Mfn2KO mice should share similar muscle defects as old control mice, which is not the case.
3. The authors attribute much of the defects in mitochondrial function and loss of muscle mass to a reduction in autophagy or mitophagy, however they make minimal attempts to measure flux through these pathways. Although potentially challenging, the authors should perform a more complete characterization of autophagic flux upon reduction of Mfn2. Furthermore, the authors should restore Mfn2 expression in deficient muscle to demonstrate that it can promote turnover of damaged mitochondria.
4. The authors need to clarify what controls were used for normalization for each experiment, particularly for Western blot results. In addition, in experiments where western blots are quantified from multiple samples, the authors should provide original images including all individual animals (i.e. in Fig 1A, 5D, 5G, 7A, 7C).
5. Authors should provide a more consistent loading control for western blots. GAPDH should not be used to normalize protein content in metabolic studies. B-actin was used for normalization for q-pcr. However, muscle expresses mainly a-actin.

Minor Concerns:

1. It is unclear whether the loss of Mfn2 expression in muscle of old mice is due to regulation of its stability or expression. The authors should measure Mfn2 gene expression.
2. In vitro experiments measuring turnover of autophagy/mitophagy proteins presented in Fig 5D should be presented as a single panel with control and Mfn2 knockdown on the same blot, as minor differences in baseline expression can alter conclusions.
3. A cytosolic loading control is needed to confirm the enrichment of mitochondrial fractions presented in Fig 5G.

1st Revision - authors' response 27 April 2016

Response to the Reviewers.

Referee #1:
The manuscript of Sebastian and co-authors describes the role for Mfn2 in sarcopenia and age-related metabolic adaptations. The authors have generated a muscle-specific Mfn2 knockout mice to address the relevance of mitochondrial dynamics in ageing sarcopenia. The authors have well characterized the KO mice at morphological, biochemical and metabolic level. The authors propose a mechanism where the reduction/ablation of Mfn2 leads to deficient mitophagy which in turn results into the accumulation of dysfunctional mitochondria. Damaged mitochondria produce ROS that activate a retrograde signal via HIF1-BNIP3 to induce mitochondrial removal via mitophagy. The present work is a well designed study with interesting findings. This enthusiasm is lowered by the fact that the authors already have characterized Mfn2 knockout mice in young age and have found a metabolic imbalance. The metabolic changes observed may indirectly induce the precocious ageing phenotypes observed in these KO mice. However, this work better clarifies some of the contradictory results present in literature. In fact, there are several reports connecting sarcopenia with dysregulation of mitochondria quality control. However, mitochondrial dynamic protein levels have been found either downregulated (Ibebunjo et al. 2012) or unchanged (Leduc-Gaudet et al. 2015). Thus, the causal link between sarcopenia and unbalanced mitochondrial fusion/fission is not clear. Therefore, this manuscript provides interesting data supporting a causative role of mitochondrial dynamics to age-related muscle loss. However, some findings are preliminary and need further experiments to sustain author's claims. The authors should consider the following points.
Response: We want to thank very sincerely the reviewer for the insightful comments. We have analyzed every single issue raised by the referee, and as a result, we honestly think that have substantially improved our manuscript.

Major Points:

Figure 1. The authors start the paper demonstrating that aging is characterized by a progressive reduction of Mfn2 protein levels (Figure 1). The authors should check whether this decrease is consequent to a transcriptional-dependent suppression or protein turnover.
Response: In order to evaluate whether the decrease in Mfn2 expression during aging was a consequence of a transcriptional suppression, we have evaluated Mfn2 mRNA levels in skeletal muscle from young and old mice. Our data show no changes in Mfn2 mRNA levels between young and old mice. In addition, we have evaluated Mfn2 mRNA levels in polysomal fractions, which contain mRNAs bound to ribosomes. Again, no changes were found between young and old mice, indicating that Mfn2 mRNA expression or distribution were not affected by age. Therefore, our results suggest that reduced levels of Mfn2 protein in old mice are not a consequence of reduced expression of Mfn2 mRNA or lower availability of Mfn2 mRNA for translation but rather due to changes in protein stability or actual translation. We have incorporated all these data in Fig EV1D and EV1E.

Moreover, It would be of interest to know whether this decrease is present in other muscles a part from gastrocnemius such as soleus, EDL, quadriceps or Tibialis Anterior.
Response: Mfn2 expression was also measured in soleus and tibialis anterior muscles showing that age leads to a reduction of Mfn2 protein expression in all skeletal muscles studied. Of note, the decrease in Mfn2 detected in soleus muscle was higher than in tibialis anterior or in

gastrocnemius, suggesting that slow-twitch fibers are affected by age to a greater extent than fast-twitch fibers. Results are shown as Figure EV1A.

The densitometric quantification of Mfn2 should be normalized for mitochondrial proteins in order to detect a relative decrease of Mfn2 compared to mitochondrial content.
As it can be seen in Figure 1A, porin or ATP5a protein levels are not changed in muscles when comparing young and old groups. This information, together with the measurement of mtDNA/nDNA in Figure 2B indicates that there are no changes in mitochondrial mass between young and old mice. In consequence, the reduced levels of Mfn2 in old mice are not a consequence of changes in mitochondrial mass. Below you will find the quantification of normalized Mfn2 expression levels relative to porin (Fig R1.1)

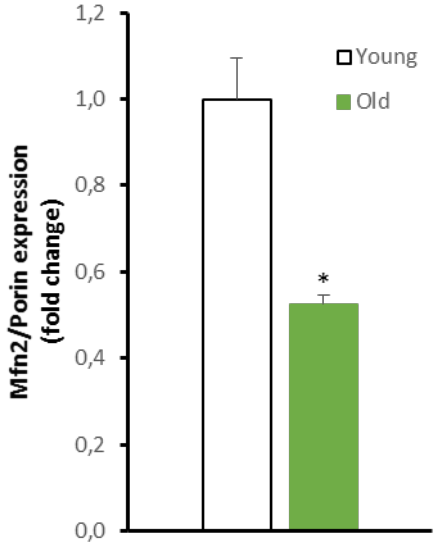

Figure R1.1. Mfn2 protein expression in gastrocnemius muscle from young and old mice. Data are normalized to porin levels (n=6 mice per group). *p<0.05 young vs. old mice.

Point 2. One critical point of this genetic model is that the deletion of Mfn2 looks not specific. Indeed authors described that Mfn2 is reduced in tissues like WAT, liver and heart. Therefore, it is difficult to say which tissue contributes to the age-related metabolic alterations. It would be important to monitor mitochondrial complex activity and mitochondrial respiration also in WAT, Liver and heart in order to have an idea whether mitochondria are compromised in these tissues.

Response: We thank the reviewer for raising this important issue that was not specifically addressed in our previous version. As the referee mentions, genetic deletion of Mfn2 is not specific for skeletal muscle, and Mfn2 protein levels are also decreased (although to a lower extent) in other tissues such as white adipose tissue (WAT), liver and heart. To further characterize the origin of metabolic alterations, we have measured mitochondrial respiration in WAT, liver and heart from old mice, and we have found no changes between control and Mfn2KO mice in any of the conditions and tissues studied. These data are now shown as Figures EV2A, EV2B and EV2C. In all, our data indicate the existence of mitochondrial alterations that affect skeletal muscle (Figure 2A) but not white adipose tissue, liver or heart (Figures EV2A, EV2B and EV2C). We think that these data strongly support the notion that the phenotype of the Mfn2 KO mice studied is the consequence of mitochondrial dysfunction of only skeletal muscle and not in other tissues.
In addition, and to further confirm the specific role of muscle Mfn2 in the phenotype observed, we include for the reviewer data obtained in a different mouse model with muscle-specific Mfn2 ablation. We have crossed MLC1-Cre mice with Mfn2LoxP mice to generate MLC1-Mfn2KO mice. This led to the specific ablation of Mfn2 in skeletal muscle (Figure R1.2), with no changes in Mfn2 expression in liver, white adipose tissue or heart. Four-month old MLC1Cre-Mfn2KO showed no alterations in glucose homeostasis, as was the case for young Mef2CCre-Mfn2KO mice (Figure R1.3). However, young ML1Cre-Mfn2KO mice

showed reduction in whole-body oxygen consumption, indicating that specific ablation of Mfn2 in skeletal muscle is sufficient to reduce whole body energy expenditure (Fig R1.4). Importantly, in order to see if specific ablation of Mfn2 in skeletal muscle could also recapitulate muscle alterations, we measured cross-sectional area of MLC1Cre-Mfn2KO young mice, and we observed a significant reduction in the size of muscle fibers compared to control mice (Fig R1.5). Thus, specific ablation of Mfn2 in skeletal muscle recapitulates the phenotype observed in Mfn2KO mice (Mef2CCre-Mfn2KO). For the sake of clarity we have not included these data in the revised version but we are open to do so if that is considered necessary.

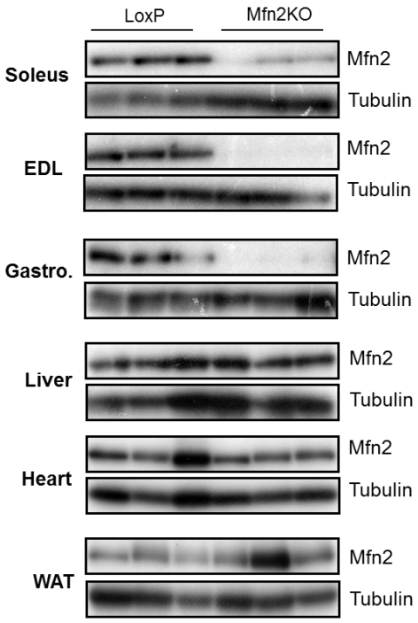

Figure R1.2. Mfn2 protein levels in different tissues from MLC1Cre-Mfn2KO mice.

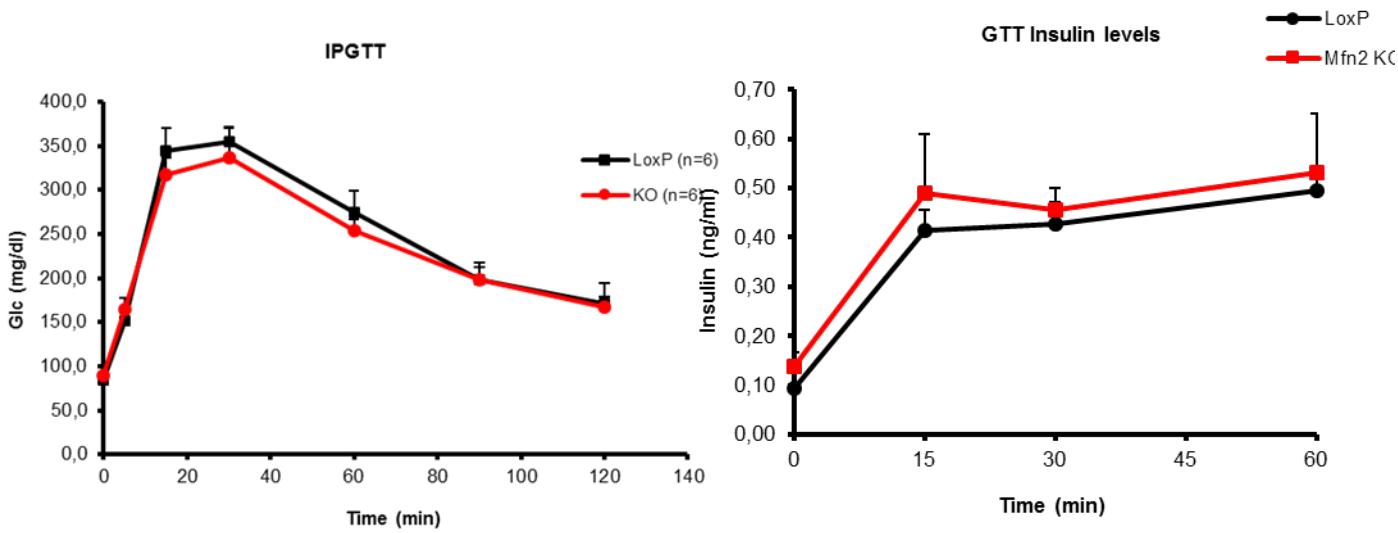

Figure R1.3. Glucose tolerance test and quantification of insulin levels during the GTT in young control and MLC1Cre-Mfn2KO mice.

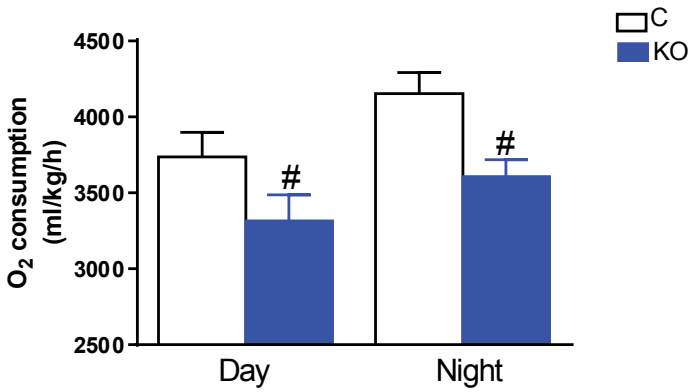

Figure R1.4. Whole-body oxygen consumption in young control and MLC1Cre-Mfn2KO mice.

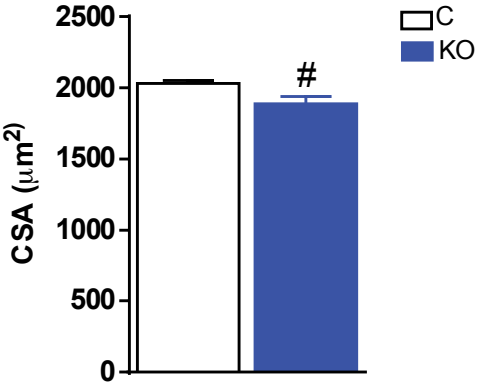

Figure R1.5. Cross-sectional area of muscle fibers from gastrocnemius muscle in young control and MLC1Cre-Mfn2KO mice.

Point3, Figure 2. During ageing it has been reported an increase of mtDNA mutation. The authors should check whether there is an increase of COX negative/SDH positive fibers in Mfn2 KO compared to controls.
Response: We have performed sequential COX-SDH staining in gastrocnemius from young and old control and Mfn2KO mice. The data revealed no changes in the number of COX negative/SDH positive fibers between groups, indicating no increase in mtDNA mutation during age or between genotypes. Results are shown as Figure EV2K.

The authors claimed that Mfn2 KO muscles contain giant mitochondrial. The EM pictures shown in panel 2G are small and it is difficult to appreciate mitochondrial morphology. Larger images are encouraged. However, looking also at the panels 2H, it does not appear that mitochondria are giant but more likely are swollen.
Response: We agree with the reviewer that the EM pictures shown in Fig 2G were small. We have replaced them for new images showing clearly that mitochondrial size is increased in old mice. Moreover, we have moved the former EM pictures in Fig 2G to Fig EV2L. These two sets of figures permit to clearly visualize that during aging and Mfn2 deficiency, there is a reduction in mitochondrial number and an increase in mitochondrial size. In addition, we also agree with the referee it is more appropriate the word swollen than giant to refer to the aberrant mitochondria shown in Fig 2G. We have changed this accordingly.

Moreover, it would be important to characterize the fission machinery. Is there any changes in DRP1/fis1/Mff proteins that would account for the giant mitochondria mentioned by the authors? What about Mfn1 and OPA1 levels in aged Mfn2 KO mice? Finally, the quantification of Mitochondrial complex activity would be an important addition to the characterization of mitochondria when mfn2 is absent.

Response: We have assessed the levels of fission proteins (Fis1 and Drp1) and other fusion proteins (Mfn1 and OPA1) in aged muscle, and we have found that Fis1, OPA1 and Mfn1 are reduced, but no Drp1 (Figure EV1F). These data point out to a general deregulation of mitochondrial dynamics during age.
Moreover, levels of Mfn1, OPA1, Drp1 and Fis1 were not altered either in young or in old mice in response to Mfn2 ablation (Figure EV1F). In all, our data suggest that the mitochondrial alterations seen in old mice are likely a consequence of a general deregulation of mitochondrial dynamics during age. However, the swollen mitochondria detected in Mfn2-deficient muscles are not a consequence of alterations in other proteins involved in mitochondrial fusion or fission.

Point 4. Figure 3. it would be interesting to monitor some of the pathways involved in glucose uptake in muscles such as Akt, AMPK, TBC1D1, AS160.
Response: Indeed, we have measured the phosphorylation and total levels of Akt and AMPK in muscles from young and old mice under control or Mfn2-deficient conditions. Data are shown in Figure EV3F, and indicate the absence of changes due to Mfn2 deficiency. Instead, phosphorylated Akt was reduced with age, whereas phosphorylated AMPK was marginally enhanced in old Mfn2KO mice.

Point 5. Figure 4. During ageing there is a progressive loss of motorneurons leading to denervation. This process mainly happens in fast fibers and is partially counteracted by reinnervation of slow motorneurons. Nerve sprouting and fiber-type grouping are typical features of ageing sarcopenia. Did the authors reveal an increase of type I fibers and/or type I-grouping?
Response: We have detected type I, IIb and IIa/IIx muscle fibers in young and old mice with the different phenotypes. The results are shown as Figure 4D, and there is no evidence for the existence of alterations associated to aging in control or Mfn2 KO mice. However, we document an increased number of type I fibers and in type IIx fibers in muscles from Mfn2 KO young mice compared to wild-type. Under these conditions no evidence for type I-grouping was documented.

Experiments represented in Figure 4 correlate Mfn2 abaltion with the reduction of muscle mass and function. Figure 4G shows that old Mfn2 KO mice have reduced muscle performance on a treadmill. However, this type of test does not discriminate between the performance of skeletal or cardiac muscles. It is worth to underline that these mice show a reduction of Mfn2 in both heart and skeletal muscles. Sarcopenia can be defined as the reduction of both muscle force and muscle mass. Importantly, the reduction of muscle force is faster than the reduction of muscle mass. Therefore, the analyses of muscle force in Mfn2 KO would be more informative than exercise performance.
Response: We thank the referee for the great suggestion. We have performed studies aimed to characterize muscle function in Mfn2KO mice. First of all, we agree with the referee that changes in muscle performance test could be also due to heart abnormalities, and this is important in our mouse model in which Mfn2 is also reduced in heart. To clarify this point, we have performed mitochondrial respiration assays in heart from old control and Mfn2KO mice, showing no differences between genotypes (Figure EV2B). That indicates, that hearts from Mfn2KO mice do not undergo metabolic alterations.
Secondly, to directly assess muscle force, we have performed *in vivo* and *ex vivo* muscle force experiments. *In vivo* muscle force was measured by grip strength test, showing a reduction of muscle strength in old Mfn2KO mice compared to controls. The results are now shown as Figure 4H.
Ex vivo muscle force measurements were also performed in soleus muscles from control and Mfn2KO mice. The data indicates a decrease in maximal tetanic force in Mfn2KO mice. Moreover, alterations normally seen during aging in mouse, rat and human muscle (Brooks and Faulkner, 1988; Campbell et al, 1973; Fitts et al, 1984) such as an increased twitch to tetanus ratio, and increase in half relaxation and contraction times were also detected in Mfn2KO old mice. These results are now shown as Figure 4I, 4J, 4K, and 4L. Together, we believe that all these data clearly demonstrates that Mfn2 deficiency promotes sarcopenia and frailty.

Muscle mass is controlled by the balance between protein degradation and protein synthesis. While the authors attempt to investigate the activation of proteolytic pathways responsible of muscle loss, protein synthesis is superficially studied. Indeed, western blot analysis of phospho- and total-4EBP1

is not sufficient to determine the level of protein synthesis. A better investigation of Akt-mTOR pathways is required. Finally, protein synthesis should be measured in sarcopenic muscles by using puromycin incorporation.

Response: Following the reviewer suggestion, we have included additional measurements to document the impact of Mfn2 deficiency on muscle protein synthesis. First, we have measured levels of phosphorylated and total levels of S6 (another downstream target of mTOR). These data indicate a reduction of pS6 during age in control mice (Figure EV4F). In addition, our data indicate a reduced S6 phosphorylation in young Mfn2-deficient muscles, which points out to a reduced protein synthesis (Figure EV4F). Therefore, we evaluated protein synthesis *in vivo* by using SUnSET method. As it is shown in Figure EV4G, puromycin incorporation was clearly reduced in Mfn2KO mice, indicating a substantial inhibition of protein synthesis. In all, our data strongly support the view that Mfn2 deficiency reduces protein synthesis in skeletal muscle, and this could be an additional mechanism of sarcopenia.

Point 6. Figure 5. The authors excluded an involvement of ubiquitin-proteasome system based on the expression levels of atrogin1 and MuRF1 and on proteasome activity, determined in vitro. However, we now know that many other ubiquitin ligases are involved in muscle atrophy. Moreover, the proteasome activity depends on the level of the poly-ubiquitinated proteins unless proteasome in inhibited. Authors should check whether the amount of poly-ubiquitinated proteins is increased in Mfn2 KO mice. A western blot for Lys48 poly-ubiquitinated proteins and its quantification would better complement the analyses of the Ubiquitin-proteasome system.
Response: We fully agree with the reviewer. To better characterize the ubiquitin-proteasome system we have measured the expression of other E3-ubiquitin ligases involved in muscle atrophy, such as *SMART*, *MUSA1* and *FbxO31* (Milan G et al, 2015). No changes in the expression of those genes were detected when comparing control and Mfn2 KO mice (Figure EV4A). No changes in the expression of SMART, MUSA1 and FbxO31 genes was detected between young and old mice.
In addition, we have measured the abundance of K48-linked ubiquitinated proteins (Figure EV4B) and no changes were found between genotypes, indicating that Mfn2 deficiency does not affect protein degradation by the ubiquitin-proteasome pathway. Under these conditions, aging was associated to a reduced level of K48-linked ubiquitinated proteins.

The authors have used C2C12 to monitor autophagy flux. The authors should confirm the in vitro data and monitor autophagy flux in vivo by colchicine or leupeptin treatments.
Response: We have assessed *in vivo* autophagy flux by injection of chloroquine in control and Mfn2 KO mice. These data are now shown as Figure 5D, and are completely coherent with data obtained in cultured muscle cells. Thus, Mfn2-deficient muscles showed a reduced build-up of LC3-II, and p62 in response to chloroquine treatment. Under these conditions, the increase of BNIP3 was similar in control and KO groups, and basal levels of BNIP3 were higher in Mfn2KO. In all, these data indicate that autophagy flux is reduced in Mfn2-deficient muscles.

The changes of p62 level displayed in panel 5B may reflect transcriptional changes. The authors must check the mRNA levels of p62/SQSTM1 in Mfn2 KO.
Response: We have measured p62 mRNA levels and no changes were found between groups. Data are shown as Figure EV5A.

In panel 5D the authors found an identical increase of LC3II band after bafilomycin treatment in control and Mfn2 KO cells. This does not happens for p62, NBR1 and BNIP3. The authors' claim that autophagy flux is reduced is incorrect. The right explanation of these data is that non-selective autophagy is maintained but this is not the case for the selective autophagy that requires the adaptor proteins p62, nbr1 and bnip3 for the selective removal of damaged proteins/mitochondria. Thus, Mfn2 KO mice upregulate non selective autophagy because the selective autophagy is impaired. It would be important to check LC3II, P62 and BNIP3 levels on purified mitochondria from muscles of Mfn2KO and controls in presence or absence of Colchicine. The experiments on purified mitochondria from myotubes shown in Fig 5G support the hypothesis of an impairment in selective autophagy but does not consider the flux.
Response: We appreciate very much the referee's suggestion. We agree with the referee that at least in C2C12 myotubes, Mfn2 deficiency seems to affect to a greater extent selective-

autophagy processes (involving p62, NBR1 and BNIP3) than to general non-selective autophagy. Accordingly, we have introduced this concept in the discussion section of the revised manuscript (page 15, 3rd paragraph).
In addition, we have measured the level of LC3II, p62 and BNIP3 in mitochondrial fractions from skeletal muscle of control and Mfn2 KO mice untreated or treated with chloroquine for 5 days. Chloroquine-induced accumulation of these proteins (LC3-II, p62 and BNIP3) in mitochondria from control mice. However, the accumulation detected upon chloroquine in the Mfn2-deficient group was markedly reduced for all three proteins studied. These data confirms the view that mitophagy flux is also inhibited in vivo in Mfn2 KO mice, and are now shown as Figure EV5C.

Figure 7F recapitulates the mechanism proposed in this work. The figure is representative of the data but the last panel is unclear. To my opinion, in this panel the line connecting Mitochondrial quality control to mitochondria should be removed, mitochondria should become swollen, the arrow coming out from mitochondria and the world ROS should be enlarged and all the arrows connecting HIF1a- Bnip3-mitchondrial quality control removed as well as the text (HIF1a, BNiP3). This new version would better explain the message that when quality control is impaired then dysfunctional mitochondria accumulate leading to enhanced ROS production. Finally, the sentence in the middle panel "Impaired metabolic homeostasis sarcopenia and muscle dysfunction" should be removed since it is also present in the last panel and makes confusion. In fact, in healthy ageing the HIF1a loop should work in order to minimize the age-related changes. Maybe the authors can use "physiological age-related muscle loss" in healthy ageing and "sarcopenia and frailty" in Unhealthy ageing.

Response: We agree with the referee that the information given in Figure 7F should be improved. We have modified the figure accordingly to make it clearer. In particular we think it is important to emphasize that ROS is enhanced, and to change the definition of the alterations. Of note, new data obtained in mice treated with NAC has shown that blockage of ROS worsened muscle atrophy (Figure EV7E). These data suggest that ROS is necessary for maintaining the BNIP3-adaptive pathway and minimize muscle damage. For that reason we think appropriate to maintain the arrows connecting ROS to the BNIP3-adaptive pathway. Again we want to thank the reviewer for the great support in improving the message of the manuscript.

Minor Points:

-Figure 1B: The comparison between genes deregulated in old Mfn2 KO mice versus old wild-type is missing.
Response: We have added a panel showing this comparison in Figure 1B.

-Figure EB2A: Western blots are not shown and should be provided.
Response: We have added a representative WB in new Figure EV2D.

-Figure 5B: Western blot for tubulin is oversaturated
Response: We have performed a new WB assay for tubulin with the same samples and we have used this image for Figure 5B.

-Figure 5G: Western blot for Parkin was cropped at the level of the band.
Response: We have changed the image.

The reduction of autophagy in aged muscles have been shown in the work of Carnio et al. Cell Reports 2014 in which authors showed that autophagy is decreased in aged humans and mice, that autophagy inhibition worsens muscle atrophy and force generation and that restoring autophagy in aged muscle ameliorates muscle mass and innervation. Therefore, this paper should be mentioned by the authors in introduction and discussion.
Response: We agree with the referee. The article by Carnio et al. is now mentioned in the results and discussion sections.

Referee #2:

EMBO J (18 oct 2015)
Mfn2 deficiency links age-related sarcopenia and impaired autophagy to activation of an adaptive mitophagy pathway
1. General summary and opinion about the principle significance of the study, its questions and findings
In this paper, the authors show that during skeletal muscle aging Mfn2 is a key molecule to regulate autophagy and mitochondria quality control. They show that Mfn2 depletion triggers an impairment of mitophagy associated with a gene signature related to aging, thereby leading to alterations in metabolic homeostasis and sarcopenia. Interestingly, they also show that these effects are attenuated by the Mfn2-depletion-dependent activation of mitophagy through an alternative pathway driven by HIF1a activation. This paper is very coherent in the presentation of the outstanding questions, the state of the art and the results obtained. The experiments are well designed and performed, both quantitatively and qualitatively. To sum up, the work presented in this paper is very original and particularly since it adds new functions to the ones already known the mitochondrial dynamic protein Mfn2, thereby extending our knowledge on mitochondrial involvement in crucial cellular and physiological pathways.
Response: We thank the reviewer's opinion of our work and the great suggestions for improvement. We have addressed the referee's concerns and modified our manuscript accordingly.

2. Specific major concerns essential to be addressed to support the conclusions.
How can authors explain that Mfn2 expression level is decreased in aging, and in particular in highly oxidative muscle fibers? Did they check if Mfn2 expression is regulated by one of the common inducers of senescence (p53, pRb, ...), or affected by ROS ?
Response: We agree with the reviewer that this was a relevant aspect to consider. We have further investigated the mechanism by which Mfn2 is downregulated during aging. We have assessed whether Mfn2 reduction during age is a consequence of transcriptional alterations by measuring Mfn2 mRNA expression (Figure EV1D). Our data indicate that Mfn2 gene expression is not affected by age. We also isolated polysomal fractions from skeletal muscle (mainly containing actively translated mRNA bound to ribosomes) and evaluated Mfn2 mRNA in those fractions. As shown in Figure EV1E, no changes were detected between young and old mice. Altogether, these data indicate that the reduction of Mfn2 during aging could be consequence of alterations in protein turnover.
We have also measured Mfn2 expression in soleus and tibialis anterior muscles showing that age leads to a reduction of Mfn2 protein expression in all skeletal muscles studied. Of note, the decrease in Mfn2 detected in soleus muscle was higher than in tibialis anterior or in gastrocnemius, suggesting that slow-twitch fibers are affected by age to a greater extent than fast-twitch fibers. Results are shown as Fig EV1A.
Regarding the role of senescence inducers on Mfn2 expression, this was evaluated in proliferative or replicative senescent MEF cells. Senescent cells showed a characteristic increased expression of Lamin A/C. Under these conditions, Mfn2 protein levels were increased in senescent cells (Fig R2.1). We conclude from here that Mfn2 is not repressed in skeletal muscle because of senescence, and other factors must be playing a regulatory role.

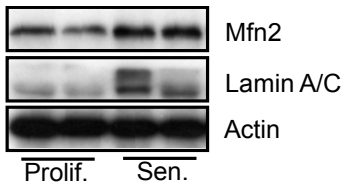

Figure R2.1. WB of Mfn2 and Lamin A/C (marker of senescence) in proliferative or replicative senescent MEF cells.

Regarding the role of ROS on expression of Mfn2, our data indicate that reduced levels of Mfn2 precedes the increase in ROS levels *in vitro* and *in vivo* (Sebastian et al, 2012, and present manuscript), because reintroduction of Mfn2 in skeletal muscle from Mfn2KO reduced ROS levels (Fig R2.2), clearly demonstrating that Mfn2 controls ROS levels.

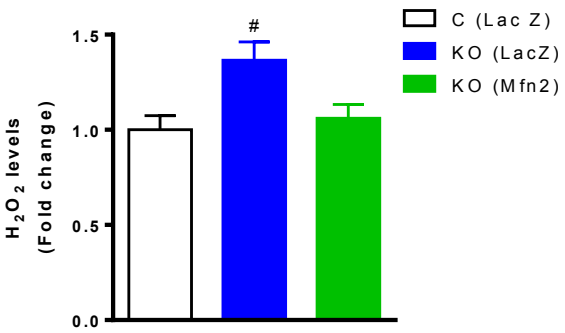

Figure R2.2. Hydrogen peroxide levels in skeletal muscle from control, Mfn2KO and Mfn2KO+Mfn2 mice.

Moreover, one experiment is missing to conclude that Mfn2 is involved in the aging process: the overexpression of Mfn2 in old mice, or at least in senescent primary cells to determine if there is a rescue in the different phenotypes observed. Furthermore, the in vitro model shall be better characterized to understand the molecular mechanisms involved. For instance, most of the in vitro experiment are performed in C2C12 cells, but the use of primary fibroblasts could be more relevant to determine if Mfn2 depletion also triggers or accelerates cellular senescence, by studying the usual markers of senescence like telomere shortening, p53, pRb, p16 or p21 activation, SA b-gal activity,...

Response: We thank the reviewer for raising this key point. We agree with the referee that in order to clearly demonstrate that Mfn2 is involved in the aging process we need to demonstrate that the reintroduction of Mfn2 in KO mice rescues the phenotype. Accordingly, we have performed this experiment in muscles of young Mfn2 KO mice. Mfn2-deficient muscles show a clear phenotype characterized by inhibition of autophagy and reduced cross-sectional area. Mfn2 gain-of-function induced by adenoviral injection into gastrocnemius muscles of Mfn2KO mice rescued Mfn2 protein levels (Figure EV5F), normalized the abundance of autophagy proteins LC3 and BNIP3 (Figure EV5G) and increased the size of muscle fibers (Figure EV5H).

As to senescence, we have analyzed some senescent markers in skeletal muscle from young and old control and Mfn2KO mice. Data are shown below as Figure R2.3. Abundance of Lamin A/C or p19ARF were not altered in Mfn2 KO mice compared to controls. A trend to reduction was detected with age although differences were not statistically significant. Based on this, we can rule out a role of senescence under our conditions.

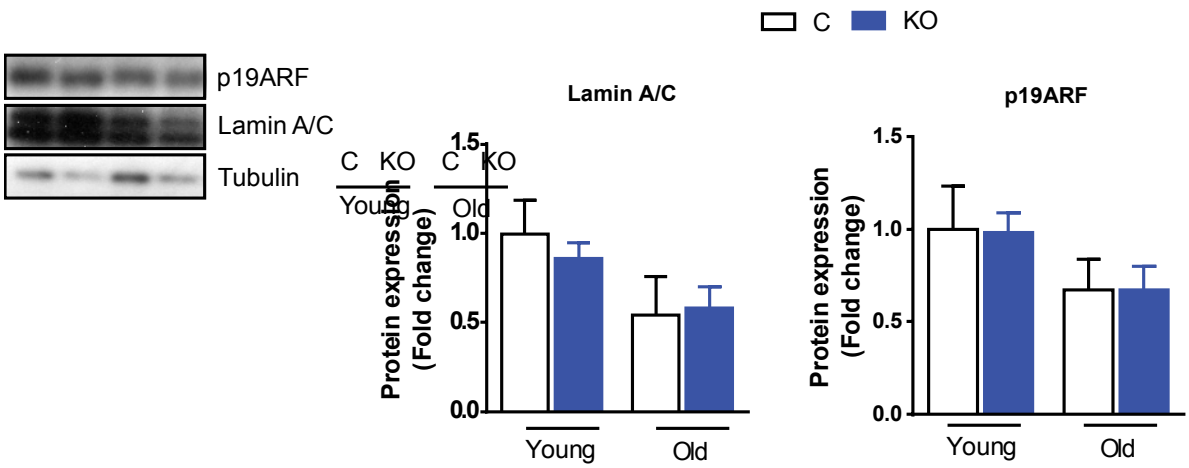

Figure R2.3. Protein expression of senescence markers in skeletal muscle from young and old control and Mfn2KO mice (n=6 mice per group).

Along the same line, the aging phenotype is caused by which of the consequences of Mfn2-ablation (or reduction)? Is it ROS production, autophagy reduction, decreased ER-mitochondria tethering (and hence autophagy) or something else? For instance, it has been shown that the inhibition of autophagosome formation by treatment with 3-methyladenine decreases the number of SA-β-GAL-positive cells upon treatment to induce senescence (Patschan et al. 2008). Is autophagy or ROS inhibition with 3MA or NAC additive to Mfn2 depletion?

Response: The reviewer raised a very important point, key to document the specific mechanism by which Mfn2 deficiency leads to muscle alterations. We have done a number of experiments to answer this point in relation to autophagy. First, we have measured autophagy flux in muscles from control and Mfn2 KO mice by treating mice with chloroquine for 5 days. Data clearly documents that Mfn2 deficiency leads to a reduction in autophagy and autophagy flux *in vivo*, and are shown as Figure 5D.

Moreover, we have analyzed the impact of inhibition of autophagy in control mice treated with chloroquine (Figure EV5E). Mice treated with chloroquine showed a reduction in muscle fiber size, i.e., an effect similar to that induced by Mfn2 deficiency (Figure EV5E). This is coherent with data obtained in control muscle cells incubated in the presence of the inhibitor of autophagy bafilomycin A, which also show effects similar to those generated by Mfn2 deficiency (Figure EV6D-H).

ROS production does not seem to be involved in the aging/Mfn2-induced muscle alterations, because treatment with NAC (which lowers ROS levels) does not rescue muscle atrophy (Fig EV7E). Importantly, reintroduction of Mfn2 in skeletal muscle of Mfn2KO mice, restored autophagy (Fig EV5C), ROS levels (Fig R2.3), and reversed muscle atrophy (Fig EV5H). Altogether, our data strongly supports the view that reduction of autophagy is the underlying mechanism by which Mfn2 reduction leads to muscle alterations.

In figure5D there are some issues. First, in the Mfn2 KD LC3 or p62 seem to be unaltered, whereas in vivo the differences between wt and Mfn2-/- were clear... How do the authors explain this discrepancy? Moreover, the quantification of BNIP3 and NRB1 levels in the experiment w/o bafilomycin does not seem to be a faithful representation of the shown WB.

Response: The reviewer raises a very interesting point. In Mfn2 KD myotubes, LC3 levels are unaltered compared to controls, both under basal and bafilomycin-treated conditions. In contrast, p62, BNIP3 and NBR1 show an enhanced abundance in Mfn2-deficient basal conditions, and less accumulation upon bafilomycin. These data are consistent with a reduction of selective autophagy (dependent of adaptor proteins such as p62, BNIP3 and NBR1) under conditions of unaltered general non-selective autophagy (LC3). However, *in vivo* in skeletal muscle, LC3II is increased in basal conditions and less accumulated in chloroquine treated conditions in Mfn2KO mice, as is p62 and BNIP3, suggesting that both non-selective and selective autophagy are decreased in Mfn2KO mice. The discrepancy for LC3 expression between in vitro and in vivo data could be explained by differences in the time of repression of Mfn2 (two days in cells and 6 months in mice) or by differences in regulation of both non-selective or selective autophagy in C2C12 cells and skeletal muscle. This concept has been introduced in the discussion section (pag15, 3rd paragraph).

Regarding the WB for BNIP3 and p62, we have included different images to better reflect the quantification data.

In figure 6, NAC is used to conclude that the ROS-dependent HIF1 pathway is important to maintain a certain level of mitophagy and counterbalance the Mfn2-depletion-dependant mitophagy decrease. However, by closely looking at the first WB, it seems that there is a huge increase in mitochondrial Parkin levels upon NAC treatment, which is not mirrored in the presented quantifications. Thus, to conclude that NAC affects the autophagic flux, Bafilomycin or chloroquine must be added. Finally, other experiments are needed to conclude that ROS production upon Mfn2 ablation is protective. What about sarcopenia, or mice activity?

Response: We appreciate very much the reviewer's suggestion to assess more deeply the role of ROS and NAC in the phenotype of Mfn2KO mice. In order to provide additional insight, we have performed autophagy flux experiments using bafilomycin in C2C12 myotubes in order to evaluate the role of NAC on autophagy. Data are shown as Figure EV7D show that NAC inhibits autophagy flux in muscle cells both in control and Mfn2 KD muscle cells. This indicates, that a certain level of ROS is necessary to sustain basal autophagy in muscle cells.

Secondly, we have used another representative image for parkin in the WB assays shown in Figure 7D, which better reflects the quantification data.
Lastly, and to further confirm that the increase in ROS is a protective mechanism, we treated mice with NAC and measured cross-sectional area. As shown in Figure EV7E, NAC treatment for 3 weeks reduced the size of muscle fibers in control mice, and the reduction was even greater in muscles from Mfn2KO mice. These data confirm that ROS has a protective role upon Mfn2 deficiency by upregulating autophagy and mitophagy.

Referee #3:

The current study by Sebastián et al. uses a model of Mitofusin 2 (Mfn2) deficiency to examine how this protein contributes to the accumulation of damaged mitochondria in skeletal muscle associated with aging and metabolic disease. The authors suggest that Mfn2 protein expression is reduced in skeletal muscle of old mice (22 months). Microarray analysis showed that many genes were similarly regulated in muscle of old control and young Mfn2 deficient as compared to young control animals (6 months). Although Mfn2 deficiency did not alter lifespan, the animals displayed many hallmarks of mitochondrial dysfunction including reduced mitochondrial density, increased ROS production and observation of large swollen mitochondria. The mitochondrial dysfunction observed in Mfn2 deficient animals was associated with development of more severe metabolic disease in old animals including reduced glucose tolerance and insulin sensitivity. Mfn2 deficient animals had smaller muscle fibers, which resulted in a reduction in muscle weight in young but not old animals. Furthermore, Mfn2 deletion was associated with reduced running capacity in old but not young animals. The authors suggest that Mfn2 knockout mice had reduced muscle autophagy that leads to atrophy and mitochondrial dysfunction. As a result, HIF1α protein levels are increased to up-regulate Bnip3-mediated autophagy.
This work confirms that aging is associated with the accumulation of damaged mitochondria and a reduced ability to repair and degrade damaged mitochondria in muscle which results in metabolic dysfunction, loss of muscle mass and activation of mitochondrial quality control pathways. Based on the data presented, it is unclear what the specific role of Mfn2 is in the regulation of this process. Furthermore, the authors are unable to uncouple the muscle-specific and systemic effects of Mfn2 deficiency on development of metabolic disease, muscle mass and muscle function. This work does not provide novel insights into the role of Mfn2 in mitochondrial homeostasis or fusion in skeletal muscle or how mitochondrial quality control is reduced by aging.

Response: We thank the critical view on our manuscript. Based on the issues raised we have performed a substantial number of experiments that are detailed below, and that provide further support to the view that the phenotype of the Mfn2 KO mice is the consequence of dysfunction that impacts in skeletal muscles and not in other tissues

Major Concerns:

1. The authors describe the animals as "muscle Mfn2-deficient mice", however they have previously demonstrated that Cre-mediated deletion of Mfn2 driven by the MEF2C promoter also occurs in many tissues including Liver, adipose tissue and brain. This group has previously demonstrated that liver specific (Albumin-Cre) deletion of Mfn2 is sufficient to cause metabolic dysfunction. A well-characterized muscle-specific promoter driven Cre such as human skeletal muscle actin 1 (hACTA1) or muscle creatine kinase (MCK) should be used.

Response: As the referee mentions, genetic deletion of Mfn2 is not specific for skeletal muscle, and Mfn2 protein levels are also decreased (although to a lower extent) in other tissues such as white adipose tissue (WAT), liver and heart. To further characterize the origin of the metabolic alterations detected in those mice, we have measured mitochondrial respiration in WAT, liver and heart from old mice, and we have found no changes between control and Mfn2KO mice in any of the conditions and tissues studied. These data are now shown as Figures EV2A, EV2B and EV2C. In all, our data indicate the existence of mitochondrial alterations that affect skeletal muscle (Figure 2A) but not white adipose tissue, liver or heart (Figures EV2A, EV2B and EV2C). We think that these data strongly support the notion that the phenotype of the Mfn2 KO mice studied is the consequence of mitochondrial dysfunction of only skeletal muscle and not in other tissues.

In addition, as suggested by the referee, we include for the reviewer data obtained in a different mouse model with muscle-specific Mfn2 ablation. We have crossed MLC1-Cre mice with Mfn2LoxP mice to generate MLC1-Mfn2KO mice. This led to the specific ablation of Mfn2 in skeletal muscle (Figure R3.1), with no changes in Mfn2 expression in liver, white adipose tissue or heart. Control mice are MLC1-Cre$^{-/-}$ Mfn2LoxP$^{+/+}$ and SkM-Mfn2KO mice are MLC1-Cre$^{+/-}$ Mfn2LoxP$^{+/+}$ mice. Four-month old MLC1Cre-Mfn2KO showed no alterations in glucose homeostasis, as was the case for young Mef2CCre-Mfn2KO mice (Figure R3.2). However, young ML1Cre-Mfn2KO mice showed reduction in whole-body oxygen consumption, indicating that specific ablation of Mfn2 in skeletal muscle is sufficient to reduce whole body energy expenditure (Fig R3.3). Importantly, in order to see if specific ablation of Mfn2 in skeletal muscle could also recapitulate muscle alterations, we measured cross-sectional area of MLC1Cre-Mfn2KO young mice, and we observed a significant reduction in the size of muscle fibers compared to control mice (Fig R3.4). Thus, specific ablation of Mfn2 in skeletal muscle recapitulates the phenotype observed in Mfn2KO mice (Mef2C-Mfn2). For the sake of clarity we have not included these data in the revised version but we are open to do so if that is considered necessary.

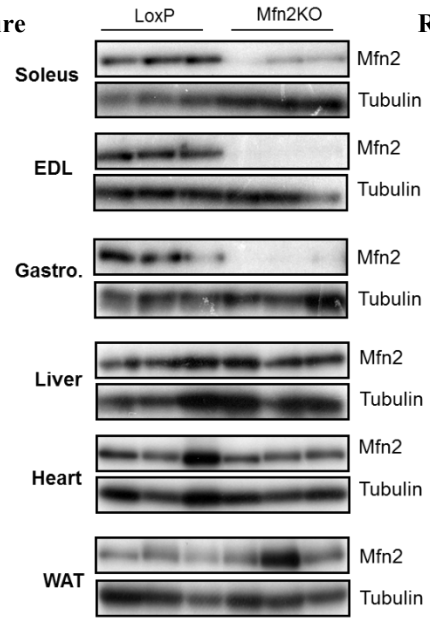

Figure R3.1. Mfn2 protein levels in different tissues from MLC1Cre-Mfn2KO mice.

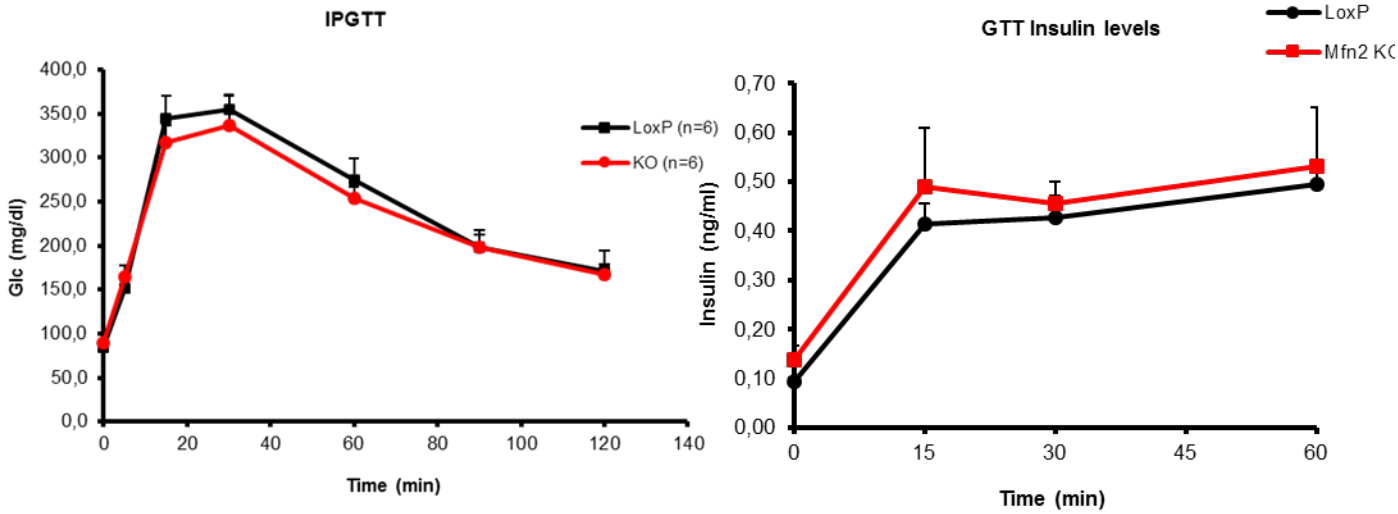

Figure R3.2. Glucose tolerance test and quantification of insulin levels during the GTT in young control and MLC1Cre-Mfn2KO mice.

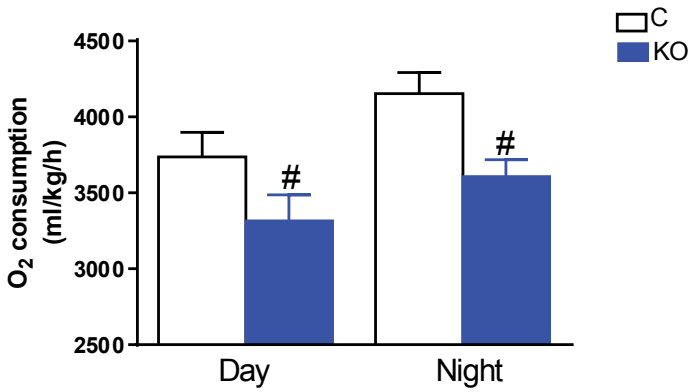

Figure R3.3. Whole-body oxygen consumption in young control and MLC1Cre-Mfn2KO mice.

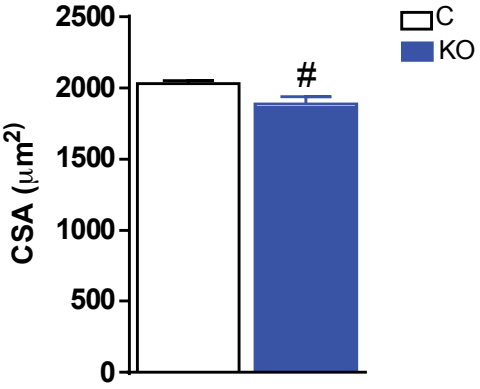

Figure R3.4. Cross-sectional area of muscle fibers from gastrocnemius muscle in young control and MLC1Cre-Mfn2KO mice.

Young animals examined in this study do not display a strong metabolic phenotype indicating that systemic insulin resistance may contribute to this phenotype. Notably, the insulin resistance phenotype in older Mfn2KO mice is likely secondary to increased weight gain. The authors should also indicate what were used as control mice.

Response: Control mice are Mef2C-Cre$^{-/-}$ Mfn2LoxP$^{+/+}$, and Mfn2KO mice are Mef2C-Cre$^{+/-}$ Mfn2LoxP$^{+/+}$. We have introduced this description in the Methods section.

As described previously (Sebastián et al, 2012), young Mfn2 KO mice do not show a strong metabolic phenotype. However, we do not believe that the increase in body weight is the cause of the metabolic phenotype in old Mfn2KO mice. This is based on: a) 1-year-old Mfn2KO mice do not show alterations in body weight compared to controls although they are already insulin resistant (Sebastián et al, 2012, present manuscript Fig EV3C-E); and b) Mfn2KO mice under a high-fat diet show a stronger insulin resistance than control mice, and this occurs in the absence of alterations in body weight (Sebastián et al, 2012). Therefore, we think that Mfn2 deficiency-induced insulin resistance is uncoupled from changes in body weight.

2. In Fig. 1B, the authors claim that Mfn2KO muscle show an aging signature expression profile. However, only a very small number of genes actually overlap with aged muscle. If Mfn2 indeed is involved in muscle aging, young Mfn2KO mice should share similar muscle defects as old control mice, which is not the case.

Response: We agree with the referee that only a small number of genes (in absolute terms) overlap between Mfn2KO muscle and aged muscle. However, what we think is relevant, is that from all the genes that change upon Mfn2 deficiency, the vast majority of them are genes that also change during aging. Therefore, this is the reason that we claim that Mfn2 deficiency creates a gene signature compatible with aging (aging gene signature).

In fact, young Mfn2 KO mice also show defects that are common to aging. Thus, there are number of alterations in young Mfn2KO mice that recapitulate those of control old mice, such as:

- **Reduction in mitochondrial function and oxidative capacity (Fig2A, 2C and 2D)**

- **Increase oxidative stress (Fig 2E, 2F)**
- **Alterations in mitochondrial size and density (Fig 2G)**
- **Reduction of muscle mass (Fig 4A)**
- **Reduction of muscle CSA (Fig 4B)**
- **Reduction in muscle performance (Fig 4G)**
- **Reduction in autophagy (Fig 5A-C)**

We now include these ideas in the discussion section (page 17, 2nd paragraph).

3. The authors attribute much of the defects in mitochondrial function and loss of muscle mass to a reduction in autophagy or mitophagy, however they make minimal attempts to measure flux through these pathways. Although potentially challenging, the authors should perform a more complete characterization of autophagic flux upon reduction of Mfn2. Furthermore, the authors should restore Mfn2 expression in deficient muscle to demonstrate that it can promote turnover of damaged mitochondria.

Response: We appreciate the referee's criticism. We have measured autophagic and mitophagic fluxes *in vivo* by injecting mice with chloroquine. As shown in Figure 5D and Figure EV5C, both autophagic and mitophagic fluxes are markedly reduced in skeletal muscle from Mfn2-deficient mice.

We have also performed rescue studies by overexpressing Mfn2 in gastrocnemius muscle of Mfn2 KO mice. Injection of adenoviruses encoding Mfn2 restored muscle Mfn2 protein levels to normal values (Figure EV5F). In these experiments, we measured the abundance of autophagy markers (Figure EV5G), and we analyzed muscle fiber size by measuring CSA in gastrocnemius muscles (Figure EV5G). Re-expression of Mfn2 restored autophagy levels, and importantly rescued muscle atrophy. These data demonstrate that deficiency of Mfn2 is the cause of reduction of autophagy (and likely accumulation of damaged mitochondria) and muscle atrophy.

4. The authors need to clarify what controls were used for normalization for each experiment, particularly for Western blot results. In addition, in experiments where western blots are quantified from multiple samples, the authors should provide original images including all individual animals (i.e. in Fig 1A, 5D, 5G, 7A, 7C).

Response: We thank the referee for this suggestion. We now include in each Figure Legend how data have been normalized. In general, we prefer providing quantification of the data coming from all animals or cell culture samples, rather than showing too many images that would make more difficult data comprehension.

5. Authors should provide a more consistent loading control for western blots. GAPDH should not be used to normalize protein content in metabolic studies. B-actin was used for normalization for q-pcr. However, muscle expresses mainly a-actin.

Response: Tubulin has been used as a loading control used for WB of skeletal muscle, as in many other reports, since β-actin gives lot of variability, likely due to the interaction with myofibrils and the contractile apparatus. However, for studies in C2C12 myotubes, we have used β-actin which gives the best results according our experience. GAPDH has only been used as a cytosolic marker to assess the purity of mitochondrial enriched fractions.

For qPCR studies, we have used both β-actin and GAPDH as a housekeeping genes, which provide reproducible results. CT values for β-actin (around 18-19 for 5 ng of cDNA) are in the range of expression compatible with its use as a housekeeping controls.

Minor Concerns:

1. It is unclear whether the loss of Mfn2 expression in muscle of old mice is due to regulation of its stability or expression. The authors should measure Mfn2 gene expression.

Response: We have measured Mfn2 gene expression and found no differences between young and old mice (Figure EV1D). Moreover, Mfn2 translation may not be affected, as suggested by the lack of alterations in Mfn2 mRNA abundance in polysomal fractions (Figure EV1E). Thus, decreased levels of Mfn2 in aged mice are likely to be a consequence of a reduced Mfn2 protein stability.

2. In vitro experiments measuring turnover of autophagy/mitophagy proteins presented in Fig 5D

should be presented as a single panel with control and Mfn2 knockdown on the same blot, as minor differences in baseline expression can alter conclusions.
Response: The images presented in old Figure 5D, Figure EV5B in the revised version come from the same blot, but extra lanes were removed for a better visualization of results.

3. A cytosolic loading control is needed to confirm the enrichment of mitochondrial fractions presented in Fig 5G.
Response: GAPDH is used as a cytosolic marker showing no visible contamination of cytosol in the mitochondrial fractions (Fig 5G of the new version).

2nd Editorial Decision 11 May 2016

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the three original referees.

As you will see, referee #1 is now broadly in favor of publication. However, as outlined by referee #3, a few amendments are still needed.
I would thus like to ask you to address the two remaining concerns and to provide a final version of your manuscript. Please note that we can add source data to figures, so please provide the western blots used for quantifications as a separate pdf file/figure to address this specific concern of the referee.

I am therefore formally returning the manuscript to you for a final round of minor revision. Once we should have received them, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

The authors addressed all my concerns. The paper is now improved.

Referee #3:

The authors performed additional experiments to address reviewers' comments. Two concerns remain:
1. The newly generated muscle-specific Mfn2KO mice are informative and the data should be included in either the main figures or supplementary figures.
2. Authors still prefer to present only "representative images" and declined the request of providing original, unprocessed Western images used for quantification. This is a requirement for many major journals now (at least in the supplementary or for review purpose). Since the quality of many Western blot results is not great, original data is particular important to support the conclusion.

2nd Revision - authors' response 25 May 2016

In the present version, we have included all the aspects you mentioned in your letter:

1. In response to Reviewer 3, we now include the data obtained in the newly generated muscle-specific Mfn2 KO mice as Appendix Figure 3.

2. Also in response to Reviewer 3, we now include a Source Data pdf File that contains all the western blots used for the quantifications found in Main Figures. The red rectangles in Source Data File are the fragments shown in Main Figures, and the black rectangles correspond to the additional experiments used in the quantification of the different Main Figures.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Antonio Zorzano
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2015-93084

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We selected a sample size of 6-7 independent observations in mouse studies in order to be able to detect statisticall differences with a statistical power values of 80%. This was assuming that we would like to detect differences of 25% of in the means of control and experimental groups and that taking into account that the SEM is aroung 10% of mean values.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We selected a sample size of 6-7 independent observations in mouse studies in order to be able to detect statisticall differences with a statistical power values of 80%. This was assuming that we would like to detect differences of 25% of in the means of control and experimental groups and that taking into account that the SEM is aroung 10% of mean values.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Only healthy mice were used in the study. In case, mice showed clearl signs of sickness, they were excluded from the study.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Mice were either young (6 months old) or 22 months old, at the time of study. Both groups showed similar body weights when they were allocated into the young or old groups.
For animal studies, include a statement about randomization even if no randomization was used.	Mice were either young (6 months old) or 22 months old, at the time of study. Both groups showed similar body weights when they were allocated into the young or old groups.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No blinding was performed.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was performed.
5. For every figure, are statistical tests justified as appropriate?	The statistical tests used were appropriate.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We analyze whether the data fit a Gaussian distribution. To this end, we perform normality test such as D'Agostini-Pearson test, Shapiro-Wilk test or Kolmogorov-Smirnov test.
Is there an estimate of variation within each group of data?	In all studies, we show the Standard Error of the Mean (SEM).
Is the variance similar between the groups that are being statistically compared?	In our experimental conditions, the variance between groups was not different. An F-test of equality of variances was routinely performed.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

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http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Mitofusin 2: ab56889 (Abcam, mouse); NBR1: ab55474 (Abcam, mouse); LC3: PM036 (MBL, rabbit polyclonal); p62 (GP62-C, Progen Biotechnik GmbH, Guinea-Pig); Tim44: t14720 (BD, mouse); VDAC-Porin: ab14734l (Abcam, mouse); alpha-tubulin: T5168 (Sigma, mouse); beta-actin: A1978 (Sigma, mouse); Bnip3: ab10433 (Abcam, mouse); Parkin: sc-32282 (SantaCruz, mouse); HIF1alpha: CAY-10006421, Cayman, Rabbit); GAPDH: ab128915 (Abcam, rabbit); p-4ebp1: 1672855S (Cell Signaling, rabbit); 4ebp1: 1679452 (Cell Signaling, rabbit); p-Akt: 1679271S (Cell Signaling, rabbit); Akt: 1679272S (Cell Signaling, rabbit); p-AMPKalpha: 1672531S (Cell Signaling, rabbit); AMPKalpha: 1672532S (Cell Signaling, rabbit); K48-linked polyubiquitin: 1674289S (Cell Signaling, rabbit); NDUFA9 (complex I): 459100 (Life Technologies, mouse); SDHA (Complex II): 459200 (Life Technologies, mouse); antioxphos CIII subunit core 1: 459140 (Life Technologies, mouse); antioxphos CIV subunit: 459600 (Life Technologies, mouse); ATP synthase subunit a (complex V): 459240 (Life Technologies, mouse); p-S6: 2211S (Cell Signaling, rabbit); S6: 2317 (Cell Signaling, mouse).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We used C2C12 mouse muscle cells tested for mycoplasma regularly. Cells were authenticated by differentiation protocols.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Male mice were used for the study. Different models have been used: C57Bl6/J wild type mice of 6 and 22 months of age; Mef2C-Cre+/- Mfn2 loxP/loxP (Mfn2KO) and Mef2C-Cre-/- Mfn2 loxP/loxP (control) mice in a mixed genetic background of SV129/C57Bl6/J of 6, 12 and 22 months of age; MLC1-Cre+/- Mfn2 LoxP/LoxP (SkM-KO) and MLC1-Cre-/- Mfn2 LoxP/LoxP (control) mice in a C57Bl6/J genetic background and 4 months of age. Mice were kept in 12hour dark-light periods, and fed standard chow diet and water ad libitum.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal work was conducted according to guidelines established by the Parc Científic de Barcelona and the University of Barcelona Committees on Animal Care.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm compliance of ARRIVE guidelines

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not applicable.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not applicable.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not applicable.

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The complete dataset for microarray data was deposited to the National Center for Biotechnology Information's Gene Expression Omnibus Database, and is accessible through GEO Series accession number GSE71501.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	We provide in Table EV1 a list of genes regulated in Mfn2-ablated muscles.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Not applicable.
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	Not applicable.
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Not applicable.

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	Not applicable.